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Scanning electron microscopic observations of the peritrophic membrane in silkworm (*Bombyx mori*) larvae

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Abstract The peritrophic membrane of the third instar larvae of a hybrid strain of the silkworm *Bombyx mori*, C146 × N137, was observed by scanning electron microscopy. The ectoperitrophic network layer was well embedded in a protein matrix, suggesting that the peritrophic membrane plays an important role in protecting the midgut cells from infection with *Bombyx mori* nucleopolyhedrovirus.

Key words Entomopoxvirus, spindle, nucleopolyhedrovirus, midgut epithelium, *Anomala cuprea*, enhancement of infectivity.

Introduction

The peritrophic membrane (PM) is a non-cellular membrane that lines the midgut lumen of insects in the form of a tube, extending from the anterior midgut to the hindgut (Derksen and Granados, 1988). The PM is composed primarily of chitin and proteins, which include glycoproteins and proteoglycans (Wang and Granados, 2001). The PM protects the midgut epithelium from mechanical damage caused by ingested materials and exposure to toxins (Sarauer *et al.*, 2003). It may also regulate the passage of materials into and out of the ectoperitrophic space and may compartmentalize the digestive processes, thus aiding the efficient sequential breakdown of ingested materials (Sarauer *et al.*, 2003). Moreover, the PM plays a role in the protection of the midgut epithelium against attack by some pathogens, including some insect viruses (Wang and Granados, 1997, 2000, 2001; Peng *et al.*, 1999; Sarauer *et al.*, 2003). For example, the PM is a barrier against the approach of nucleopolyhedroviruses (NPVs) of the family Baculoviridae, towards the microvilli of the midgut cells, which are the initial site of infection by the virus in some lepidopteran insects (*i. e.* some species of the family Noctuidae and a species of the family Arctiidae) (Wang and Granados, 1997, 2000, 2001; Peng *et al.*, 1999).

There have been few electron microscopic observations of the morphology of the PM in the silkworm *Bombyx mori* (family Bombycidae), despite the estimation that the PM in the species could have the important roles mentioned above (Rao *et al.*, 2004). Our special concern is the degree to which the PM in *B. mori* larvae is protective against *Bombyx mori* NPV (BmNPV). *B. mori* is susceptible to BmNPV, and the infectivity of the virus is markedly enhanced in *B. mori* larvae (hybrid strains N137 × C146 and C146 × N137; race Habataki) by proteinaceous paracrystalline structures, called spindles, that are produced by an insect virus, the *Anomala cuprea* entomopoxvirus (AcEPV), when BmNPV and the spindles were co-administered orally (Mitsuhashi et al., 1998; Mitsuhashi and Miyamoto, 2003). Thus the spindles are potential synergists of NPV insecticides. Mitsuhashi and Miyamoto (2003) showed that the PM of *B. mori* (hybrid strain C146 × N137) larvae was dramatically disintegrated by peroral administration of AcEPV spindles. This strongly suggested that the disintegration of the PM induced by spindles is the main cause of the spin-

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dles' enhancement of BmNPV infection in *B. mori*. The enhancement should occur because the disintegration of the PM and thus the reduction of its effectiveness as a barrier against NPV virions enable more BmNPV virions to reach the microvillus membrane of the midgut cells than when the PM is normal. However, the normal PM in *B. mori* larvae was not observed by electron microscopy in the study by our group (Mitsuhashi and Miyamoto, 2003). Electron microscopic examination would enable us to elucidate the pore sizes and their frequency of occurrence on the PM and thus to estimate to what degree the normal PM in *B. mori* larvae protects against pathogenic microbes, including BmNPV. A PM that harbors larger pores tends to allow insect viruses to pass easily to the midgut epithelium. Because some species have large-pored PMs, the PM is not considered to play an important role in protection of the midgut epithelium from infection by some insect viruses, including some NPVs (Tanada and Kaya, 1993; Mitsuhashi *et al.*, 2006). Rao *et al.* (2004) showed by scanning electron microscopy (SEM) that the pores in the PM of *B. mori* larvae are smaller than those of insect species whose PMs are not considered to act as barriers against insect viruses, at least at a certain level (Tanada and Kaya, 1993; Boucias and Pendland, 1998; Mitsuhashi *et al.*, 2006), suggesting that the PM at least constitutes a certain level of barrier against insect viruses. Nevertheless, Rao *et al.* (2004) did not disclose the name of the race that they used, and their observations were not made at high magnifications. No reports have demonstrated the pore size or distribution of pores on the PM in *B. mori* (Terra, 2001). Thus, a detailed assessment of the level of the protection provided by the PM in this species has not yet been made. Therefore, it is significant to conduct electron microscopic observations on the PM in the *B. mori* race used by Mitsuhashi and Miyamoto (2003) at various magnifications, including ones higher than those used by Rao *et al.* (2004), to estimate the level of protection provided by the normal PM in *B. mori* larvae against BmNPV.

Here, we present the results of our SEM study on the structure of the PM in a hybrid strain of *B. mori* larvae and discuss the protective role of the PM against infection by BmNPV.

Materials and methods

Silkworms

We used a hybrid strain of *B. mori*, C146 × N137, which has been maintained at our institute. The larvae were reared on an artificial diet (Silkmate; Nihon-nosan Co. Ltd, Tokyo, Japan) at 25°C until use.

SEM of the PM of *B. mori*

Four larvae at the first day of the third instar were individually transferred to wells of six-well plates (MS-8006R, Sumitomo Bakelite Co. Ltd, Tokyo, Japan), on which small pieces (each piece 3 × 5 × 2 mm) of an artificial diet (Silkmate) had been placed (one piece/well). After 36 h (*i. e.* on the third day of the third instar), three larvae that had fed completely on the diet were placed for 20 min in cold water containing crushed ice to paralyze them by chilling. The same treatment was given after 5 h (*i. e.* on the first day of the third instar) to one larva that had fed on only a little of the diet. Using a pair of forceps, the PM was carefully dissected from each larva in PBS (phosphate-buffered saline; 0.01 M Na_2HPO_4–NaH_2PO_4, 0.15 M NaCl) or sterile distilled water, rinsed in 0.1 M sodium cacodylate buffer, and then fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer. The sample was dehydrated with an ethanol series (50%, 70%, 90%, 100%), and the ethanol was then replaced with isoamyl acetate. The resulting samples were critical-point dried using a dryer (JCPD-5, JEOL, Tokyo, Japan), sputter-coated with osmium using an osmium plasma coater (NL-OPC80, JEOL DATUM, Tokyo, Japan), and then viewed by SEM (JSM-6301F, JEOL).

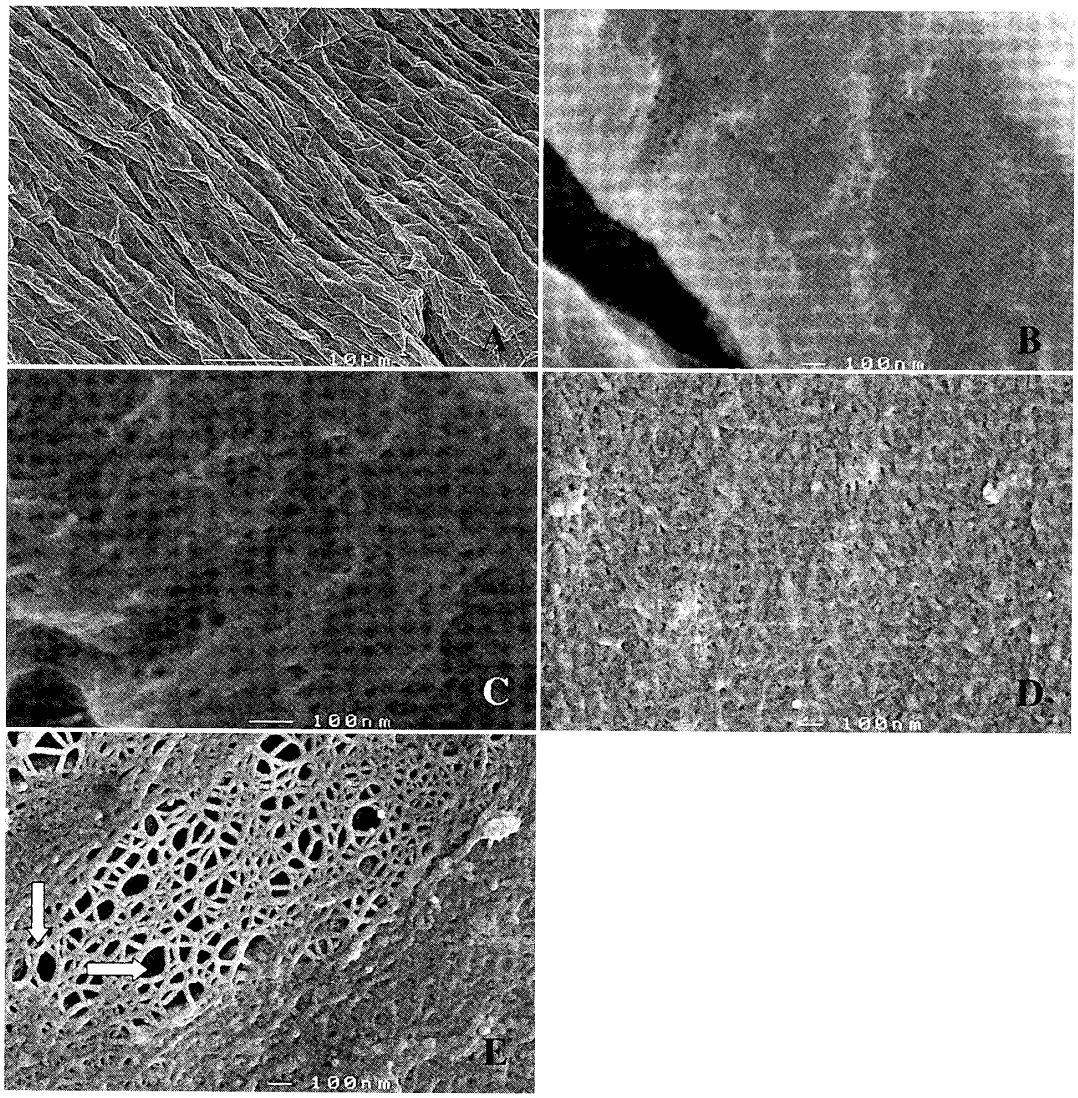

Fig. 1. Scanning electron micrographs of the peritrophic membrane (PM) of *Bombyx mori* third instar larvae. A–C: the PMs of larvae at the third day of the third instar. D and E: those of a larva at the first day of the third instar. All are the ectoperitrophic surfaces. Most of the pores were smaller than 30 nm. As shown in A, a striated structure was found when the PM was observed at relatively low magnifications. In the PM shown in E, part of the ectoperitrophic network layer has been exposed by the loss of protein matrix in which it is embedded. This loss of protein may have occurred in the course of preparation of the PM sample for SEM. The inner network inside the pores is indicated by the arrows. The pores of the exposed ectoperitrophic network vary in shape and size.

Results and discussion

All the PMs examined by SEM were very similar. Numerous pores were observed on the surface of the PM, most smaller than 30 nm (Figs 1B–D). Thus, the PM in this race harbors far smaller pores than those in larvae of some insect species described above whose PM does not play an important role as a barrier against NPVs. The pores were located in the protein matrix in which the ectoperitrophic network layer was embedded (Fig. 1). BmNPV virions are 35–45 $\times$ 300–330 nm in size (Hukuhara, 1979). The network inside the protein

matrix was multilayered, as also reported by Yamazaki (1955) (Fig. 1E). Therefore, BmNPV virions were not considered to pass readily through the PM. These structural findings strongly suggest that the PM functions to a considerable degree as a barrier against NPV virions. However, increased amounts of some BmNPV-gene products (*e. g.* enhancin), could disrupt the structure of the PM and thus allow greater numbers of baculovirus virions to pass through the PM; thus, the intake of an increased number of virions into the midgut would diminish the protective role of the PM (Lepore *et al.*, 1996; Wang and Granados, 1997, 2001).

There was a difference in the surface of the PM between the larvae at the third day of the third instar and the larva at the first day; the PM in the latter appeared thinner and its surface appeared rougher. The network within the protein matrix was more clearly detectable, since there were larger bulges in the proteins covering the network (Figs 1D and E). This difference may have been due to the difference in age of the larvae after the second ecdysis.

The pores of the network varied in shape and size, and a small proportion was hexagonal or circular (Fig. 1E), while these shapes are commonly observed in those of the larvae of the coleopteran species, *A. cuprea* (Mitsuhashi *et al.*, 2006).

The species *B. mori* is divided into a number of races, and its susceptibility to BmNPV varies among these races (Furuta, 1994, 1995). Therefore, it is possible that the ultrastructure of the PM varies among these races with respect to pore size (and hence susceptibility to BmNPV). It would be interesting to examine the PMs of races other than the one used in the present study.

Our results strongly suggest that the PM in *B. mori* (C146 $\times$ N137) can protect against BmNPV to a considerable degree. By using another method such as flux measurements (Peng *et al.*, 1999), it will be possible to demonstrate in detail our conclusion that the PM in the *B. mori* race plays an important role in protection against the passage of BmNPV through the ectoperitrophic space.

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摘　　要

走査型電子顕微鏡によるカイコ幼虫囲食膜の観察 (三橋　渡・村上理都子)

カイコ幼虫囲食膜の電子顕微鏡レベルでの観察報告がほとんどないために, 昆虫ウイルス等病原微生物の同虫への感染成立および同虫品種間でのカイコ核多角体病ウイルス (BmNPV) への感受性の差異における囲食膜の重要度は不明である. このため, カイコ交雑品種 (C146 × N137) 3齢幼虫の囲食膜を走査型電子顕微鏡で観察し, BmNPV等の囲食膜通過の難易を検討した. 皮膜細胞層側表面はタンパク層がよく発達してキチン編み目状構造の孔をほぼ埋めているために孔が小さく, この発達が悪いために孔が大きくてウイルス通過が容易とされる一部ヤガ科等昆虫のものとは明確に異なっており, BmNPVに対してかなりの程度の防御能を有するものと考えられた. 脱皮後の日齢が0日の幼虫の囲食膜では3日のものに比較して皮膜細胞層側の表面のタンパク層が薄いと思われたが, これが脱皮後初期のカイコ幼虫の一般的傾向である可能性がある. また, キチン編み目状構造の孔の形, 大きさはコガネムシの一種であるドウガネブイブイと比較して変異に富んでいた.

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